# PREPARATION AND CHARACTERIZATION OF HAP COATED- CHITOSAN-ALGINATE PEC POROUS SCAFFOLD FOR BONE TISSUE ENGINEERING

A THESIS SUBMITTED IN PARTIAL FULFILMENT

OF THE REQUIREMENT FOR THE DEGREE OF

**Master of technology**

**In**

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**By**

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# CERTIFICATE

This is to certify that thesis entitled "**Preparation and characterization of HAp coated Chitosan-Alginate PEC porous scaffold for bone tissue engineering**" by **Miss Trupti Patil**, submitted to the National Institute of Technology, Rourkela for the Degree of Master of Technology is a record of bonafide research work, carried out by her in the Department of Biotechnology and Medical Engineering under my supervision and guidance. To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University/ Institute for the award of any Degree or Diploma.

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Thanking you,

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# Nomenclature

| | | |
|---|---|---|
| I. | BTE | Bone tissue engineering |
| II. | CS | Chitosan |
| III. | AG | Alginate |
| IV. | PEC | Polyelectrolyte complex |
| V. | CAPEC | Chitosan-alginate polyelectrolyte complex |
| VI. | HAp | Hydroxyapatite |
| VII. | CAPEC/HAp | HAp coated-Chitosan-alginate polyelectrolyte complex |

# Abbreviations

| | |
|---|---|
| XRD | X-ray Diffraction |
| DLS | Dynamic Light Scattering |
| FTIR | Fourier Transform Infrared Spectroscopy |
| SEM | Scanning Electron Microscopy |
| PBS | Phosphate Buffered Saline |

# ABSTRACT

This thesis reports the development of 3D porous hybrid scaffold using chitosan-alginate polyelectrolyte complex (CAPEC) by freeze drying method. The CaCl2 cross-linked CAPEC scaffold was further coated with synthesized HAp by dip coating technique. The HAp synthesis was done by wet chemical precipitation method in organic solvent i.e. ethanol. The average particle size of synthesized HAp was found to be ~159 nm. The CAPEC and HAp coated-CAPEC (CAPEC/HAp) scaffolds were assessed for their porosity, pore size, morphology, mechanical strength, swelling behavior, in-vitro biodegradation. The pore size and morphology of developed scaffolds was studied by scanning electron microscopy (SEM) & no apparent change in pore size was found in case of CAPEC/HAp. Both the scaffolds possessed desired pore size with interconnected pore network. Whereas, the porosity was decreased with the increase in HAp coating, but it is still high enough for bone tissue engineering application. The mechanical strength of CAPEC scaffold increased with the coating of HAp, compressive strength was increased from 0.469 MPa to 0.61 & 0.67 MPa. The CAPEC/HAp scaffolds also exhibited favorable swelling behavior and biodegradation. Overall, the study has demonstrated that for non-load bearing bone tissue engineering applications, the developed CAPEC/HAp scaffolds do have the potential use.

# 1. INTRODUCTION

Bone is a dynamic and highly vascularized tissue which forms the foundation of our bodily locomotion[1]. The main role of bone is to provide structural support for the body. It also provides protection for our internal organs and load bearing capacity to skeleton[2]. Hence, Trauma, Injury or bone related diseases causing impairment or loss of bone tissue leads to reduced quality of life in many patients[3]. Growing knowledge of bone related abnormalities has permitted diagnosis capabilities and therapeutic solutions[4] which involve utilization of bone grafts to escalate bone repair and regeneration. Whereas, these treatments are associated with various issues such as donor site morbidity, limited availability, immune rejection, and pathogen transfer[1]. Failure rates in these techniques has prompted a lot of research interest among the scientific community for an alternative solution[5]. It is in this context that Bone Tissue Engineering (BTE) has emerged as an alternative strategy to treat bone abnormalities through the development of a biologically active substitute so called tissue engineered scaffold[6]. BTE typically employs the coordinated manipulation of cells, biodegradable, biomimetic scaffolds and biologically active signaling molecules[7]. A biodegradable scaffold is a 3D matrix which is inserted into the site of defect or lost bone. It supports and encourage bone tissue regeneration while it gradually degrades and is replaced by new bone tissue[8]. For success of BTE, scaffolds should have optimum porosity, appropriate pore size and interconnected pores for the passaging cells, nutrients, metabolites and signal molecules. Also, the scaffold should be nonimmunogenic, nontoxic, biodegradable at ideal rate corresponding to the rate of new tissue formation and biocompatible [9]. Besides, it should be structurally stable

and capable of providing desired mechanical strength and temporary mechanical integrity. Furthermore, scaffold must facilitate bone formation by stimulating cell adhesion, proliferation and regulating osteogenic differentiation of host cells [3, 10, 11]. In this context, material properties and appropriate fabrication technique are prerequisites for the development of 3D scaffold for bone tissue regeneration.

For BTE scaffolds, both polymers and bioactive ceramics have been developed and analyzed. Chemical composition of bioactive ceramics resembles the natural bone composition; they also promote osteogenesis and are capable of making bonds with host bone. But, clinical use of bioceramics is limited due to their brittleness and low biodegradation rate. On the other hand, biopolymers have some distinct advantages over bioceramics. Biopolymers display mechanical properties compatible with human cancellous bone. They can be fabricated easily into desired shapes and can be modified to certain extent to optimize their biodegradation rate and mechanical properties for specific applications [1, 8]. Even so, biopolymeric scaffold fail to retain their shape in long term. Development of an interconnected bioceramic-biopolymer scaffold takes advantages of both the components to meet mechanical and physiological requirements of the host tissue[12].

Over the last two decades chitosan being a natural polymer has played major role in bone tissue engineering. It is also known as best bioactive material, as it is biodegradable, bioactive and osteoconductive [11]. Next to chitosan, alginate, another natural polysaccharide is the most widely used biomaterial in the field of bone tissue engineering [13]. Although, Chitosan and alginate are generally accepted biomaterials, due to their low mechanical strength and incompetency of retaining shape for long period of time, they somehow fail as ultimate biomaterials for bone tissue engineering [8]. Being oppositely charged, chitosan spontaneously

associates with alginates in solution to form polyelectrolyte complexes (PECs) upon mixing [14]. 3D porous scaffold can be fabricated using chitosan- alginate PECs using freeze drying technique [8]. Scaffolds developed from chitosan-alginate PECs show significant improvement in mechanical and biological properties when compared to its counterparts[8]. Hence they are assumed to be having potential for bone tissue engineering purpose.

To further improve the mechanical and biological properties of scaffold and facilitate osseoconduction, biologic coating can be used. Hydroxyapatite (HAp) being chemically similar to the apatite of human bone, provide osteoconductive approach augments new bone tissue formation [15]. A very simple, energy and time saving technique, dip coating can be used for coating HAp on chitosan-alginate PEC scaffold.

In this strategy, in the present work chitosan-alginate PEC scaffolds coated with HAp will be fabricated by applying freeze drying and dip coating method. The developed scaffolds will then be characterized to study their potential for bone tissue engineering.

# 2. LITERATURE REVIEW

## 2.1 Bone tissue engineering

Bone is a dynamic and highly vascularized tissue which forms the foundation of our bodily locomotion[1]. The main role of bone is to provide structural support for the body. It also provides protection for our internal organs and load bearing capacity to skeleton[2]. Hence, Trauma, Injury or bone related diseases causing impairment or loss of bone tissue leads to reduced quality of life in many patients[3]. Growing knowledge of bone related abnormalities has permitted diagnosis capabilities and therapeutic solutions[4] which involve utilization of bone grafts to stimulate bone repair and regeneration. Currently, over 400,000 and 600,000 bone grafting operations are performed in Europe and America, respectively and the need of bone grafts is increasing[16]. Clinical practices have shown that autografts being non-immunogenic and histocompatible, serve as an excellent bone graft[1].Whereas, these treatments are associated with major concerns such as donor site morbidity, limited availability. Allografts and xenografts may raise other issues such as immune rejection, and pathogen transfer[1]. Increasing need of bone grafts has also resulted in developing bone implants. Traditional porous bone implants are made of ceramics and metals. But these implants are unable to integrate properly with the host tissue, resulting in poor surgical outcome. Other reasons for unsatisfactory outcomes involve mechanical mismatch, corrosion and wear[17]. Failure rates in these techniques has prompted a lot of research interest among the scientific community for an alternative solution[5]. It is in this context that Bone Tissue Engineering (BTE) has emerged as an alternative strategy to treat bone abnormalities through the development of a biologically active substitute so called tissue

engineered scaffold[6]. BTE typically employs the coordinated manipulation of cells, biodegradable, biomimetic scaffolds and biologically active signaling molecules[7].

### 2.2 Biodegradable scaffolds

A biodegradable scaffold serves as a temporary skeleton in bone tissue engineering. It is implanted into the defect site in order to support and encourage bone tissue regeneration. As the new bone tissue is formed, a biodegradable scaffold gradually degrades being replaced by newly formed tissue [1]. A scaffold must provide a porous network for the transport of nutrients, signal molecules, metabolite and cells. It should be biocompatible and rate of biodegradation should be equal to the rate of new tissue formation[9]. Besides, it should be structurally stable and capable of providing desired mechanical strength and temporary mechanical integrity. Furthermore, scaffold must facilitate bone formation by stimulating cell adhesion, proliferation and regulating osteogenic differentiation of host cells [3, 10, 11].

Following are the requirements of an ideal scaffold in bone tissue engineering,

I. Biocompatibility:

   A scaffold should be nontoxic and nonimmunogenic to the host tissue and it should be able to support normal cellular activities, cell signaling. It should allow cell adhesion, proliferation and formation of extracellular matrix on its framework. In addition to being osteoconductive, an ideal scaffold should also be osteoinductive i.e. it should be able to induce new bone tissue formation.

II. Mechanical Properties:

   An ideal scaffold must have mechanical properties equivalent to host bone properties. As bone is a very complex tissue with a complex biomechanical system, its mechanical

properties vary widely from cancellous to cortical bone. Compressive strength and young's modulus of cortical bone is between 100-200 MPa and 15-20 GPa, respectively. Whereas in case of cancellous bone it varies from 2-20 Mpa and 0.1-2 GPa. It is difficult to design an ideal bone scaffold due to this large variation in the mechanical properties of bone tissue.

III. Pore size:

For bone tissue engineering, an ideal scaffold should have pore size of at least 100μm. However, scaffolds with pore size range of 200-350μm are found to be optimum for bone tissue engineering. Micropores are essential for diffusion of nutrients, cell signaling molecules whereas macro pores allow tissue ingrowth. It has been reported that scaffolds with both micro and macro pores perform better than only macroporous scaffold. Unfortunately, mechanical properties reduce with the increase in the porosity.

IV. Bioresorbability:

An ideal scaffold should be able to degrade with time in-vivo at the same rate as new tissue formation. It should be resorbed at a controlled rate, creating space for new bone tissue growth. The degradation behavior of the scaffold varies depending on the application (site of implant).

Hence, key challenge in bone tissue engineering is to design and manufacture a porous scaffold with ideal composition, mechanical properties and biodegradability.

### 2.2.1 Polymeric scaffolds

Biopolymers are known to be bioactive, biocompatible and biodegradable. Polymers can be processed and tailored to get porous or fibrous scaffolds. Natural polymers such as Alginate,

chitosan, silk, collagen, hyaluronic acid and fibrin are commonly used in bone tissue engineering. Poly-lactic acid, poly-glycolic acid and poly-caprolactone are some of the synthetic polymers used [12].

### 2.2.2 Hybrid scaffolds

It is well known that any biomaterial cannot satisfy the requirements of bone scaffolds. Hence, efforts have been made in developing hybrid scaffolds. Hybrid scaffolds can be formed by combining two or more biomaterials. This allows designing a scaffold with enhanced properties. The scaffold can be made up of polymer-polymer blend or polymer-ceramic composite. Polymer-polymer blends can be formed by mixing two or more polymers forming a miscible blend with enhanced properties. It is reported that chitosan-alginate[8], chitosan-pectin-alginate[18] blends have been used to design scaffolds for bone tissue engineering. Polymer-ceramic composites are prepared by mixing a polymer with an inorganic ceramic material. Composites of hydroxyapatite with polymers such as chitosan[19], alginate[13], PLA, gelatin, collagen[1] have been reported to be successful in bone tissue engineering.

## 2.3 Chitosan and alginate as ideal biopolymer for tissue engineering

Chitosan is a natural biopolymer found in cell walls of fungi and shells of marine crustaceans. It is a copolymer of β-(1→4)-2-acetamido-d-glucose and β-(1→4)-2-amino-D-glucose unit linkages. Chitosan is considered to be having excellent biocompatibility and bioactivity[8]. it also has an intrinsic antibacterial property. Chitosan possesses hydrophilic surface which helps in cell adhesion, proliferation, and differentiation. It has also shown to promote bone tissue growth and mineral deposition by osteoblast culture. Chitosan is highly capable of forming porous structure through lyophilization. It can also be tailored into gel , beads, sponges, fibers[19]. In

spite of its general acceptance as a tissue biocompatible material, chitosan is mechanically weak and unstable, and unable to maintain a predefined shape for transplantation as a result of swelling[8]. Hence to overcome the limitations of pure chitosan scaffolds and to develop scaffolds with enhanced properties, chitosan is combined with other polymers such as alginate, hyaluronic acid, PLA or bioceramic like hydroxyapatite, calcium phosphate[11].

Next to chitosan, Alginate is the most widely used natural biopolymer in bone tissue engineering. It is obtained typically from brown seaweed including L. hyperborea, L. digitata, L. japonica, Macrocystis pyrifera, and Ascophyllum nodosum. Due to alginate's excellent biocompatibility, nontoxicity, relative low cost it has found many biomedical applications [20]. Alginate is widely used as an instant gel for bone tissue engineering [2]. Also, it can be easily modified to form hydrogels, sponges, foams, microspheres, and fibers [13]. Alginate gels can be used to deliver osteoinductive factors, bone forming cells or both for bone regeneration[20]. However, alginate gels do not possess sufficient mechanical strength for load bearing applications. Hydroxyapatite can be added to alginate gels to improve the mechanical properties as well as to enhance bone tissue formation[13].

**2.4 Chitosan-alginate polyelectrolyte complex (CAPEC)**

Although, Chitosan and alginate are generally accepted biomaterials for bone tissue engineering, they possess low mechanical strength and they are incompetent of retaining shape for long period of time. To combine their individual properties, Chitosan-alginate composites are developed. This composite is one of the most studied materials in bone tissue repair.

Chitosan is positively charged at low pH values (below its pKa value), whereas alginate is an anionic polymer. Being oppositely charged, when mixed with each other they spontaneously

associate to form polyelectrolyte complexes [3]. The polyelectrolyte complex (PEC) is formed through ionic interaction between protonated amines on chitosan and carboxylate moieties on alginate. PECs can be formed in the form of membrane [21], capsule [22], fiber [23], and scaffold [24].

The formation and stability of these PECs depend on many factors such as the degree of ionization of chitosan and alginate, charge density, the charge distribution over the polymeric chains, polymer concentration, their mixing order, the mixing ratio, the duration of the interaction, molecular weight of the polyelectrolytes as well as the temperature, ionic strength and pH of the reaction medium [3]. Biodegradation studies on PECs have showed the effect of lysozyme on the PEC is negligible. PECs have a high ability of lysozyme adsorption, but due to strong interaction between chitosan and alginate polymeric chain enzymatic hydrolysis is hindered[21]. The rate of biodegradation may be regulated by changing the polymer ratio, it indicates that PECs has high potential for scaffolds in tissue engineering.

It is also reported that scaffolds developed from CAPECs have higher mechanical strength than the individual polymer scaffolds. CAPECs can be fabricated into a scaffold with very high porosities and high interconnectivity. Also crosslinking with calcium ions provide more rigidity to the scaffold, this allows the scaffold to absorb solution without considerable swelling[22].

Thus scaffolds developed from chitosan-alginate PECs are assumed to be having potential for bone tissue engineering purpose.

**2.5 Preparation of CAPEC hybrid Scaffold**

Using Chitosan-alginate, porous scaffold as well as fibrous scaffolds can be fabricated. Porous scaffold can be prepared through a simple lyophilization technique [8, 9, 18, 23, 24]whereas

preparation of fibrous scaffold involves various techniques like wet spinning, spray spinning to produce the PEC fibers and then lyophilization of these fibers to get 3D scaffold[25, 26].

### 2.5.1 Chitosan-alginate porous scaffolds

Chitosan-alginate porous scaffolds can be fabricated by various methods. A general method involves gradual mixing of the two components to form PECs and using these PECs, a scaffold is fabricated. Other methods involve fabrication of a framework of either component followed by immerging it into the other components solution to facilitate the formation of PEC. These methods can be described as follows:

I. Scaffold fabrication using PECs formed by gradual mixing of Chitosan and Alginate,

II. Immersion of Alginate scaffold in Chitosan solution leading to PEC formation (Alginate-chitosan PEC Scaffold),

III. Immersion of Chitosan scaffold in Alginate solution leading to PEC formation (Chitosan-alginate PEC Scaffold).

*Scaffold fabrication using PECs*

This method is involves the formation of PECs first, followed by fabrication of scaffold by freeze drying the PECs. PEC formation is achieved by blending the chitosan and alginate solutions together. Order of addition of one polymer into other, blending conditions, pH of the solution, the ionic strength and temperature affect the size of PECs formed. By optimizing these conditions PECs of nanometer to micrometer range can be produced. To increase the mechanical strength of the scaffold made, it is cross-linked using Calcium chloride solution. This results in the crosslinking of the alginates present in PECs enhancing the structural stability and

mechanical strength. After crosslinking, the scaffold is freezed again and lyophilized. It has been reported that scaffold fabricated with this technique has significantly improved mechanical strength than its chitosan counterpart. Also ~92% porosity and a compressive modulus of 8.16MPa and yield strength of 0.46 MPa, respectively can be achieved. The scaffolds were found to be osteoconductive [8].

*Alginate-Chitosan PEC scaffold*

Pure alginate scaffold is immersed in chitosan solution to allow on site PEC formation. This immersion is then lyophilized to get alginate-chitosan PEC scaffold with interconnected pores. To further increase the mechanical strength and structural stability of the scaffold, it is crosslinked using calcium chloride. It is reported that, The AG–CS PEC scaffold fabricated through above method exhibites higher compressive strength compared with pure alginate scaffold[23].

*Chitosan-Alginate PEC scaffold*

In this method chitosan scaffold is formed first and then it is immersed in alginate solution facilitating the formation of PEC. After dipping the scaffold in alginate, it is freezed and subsequently lyophilized. This method allows uniform PEC formation without destroying fine pore structure of the scaffold. It is also shown to be activating the production of mineralized matrix from the cells [24].

### 2.5.2 Chitosan-Alginate Fibrous scaffold

Chitosan-Alginate fibrous scaffold can be fabricated by extruding one polymer solution from a nozzle into other polymer solution to formulate fibers. These fibers are then freeze dried to get the scaffold.

In spray-spinning, alginate solution is agitated continuously using magnetic stirrer while chitosan solution is being sprayed into it. Because of the agitation the sprayed chitosan solution is sheared into streamline. This results in the formation of elongated fibers of chitosan-alginate PEC. The spray-spun fibers thus formed are then centrifuged and washed. The collected fibers are fabricated into a 3D scaffolds by freeze drying them[26]. Spray spun fibrous scaffold has been reported to be investigated for its potential use as connective tissue regeneration. But it can be optimized further for applications of BTE.

In wet spinning, alginate fibers are produced first with the help of calcium chloride crosslinker. These alginate fibers are then immersed in the chitosan solution. The chitosan coating results in the formation of an alginate-chitosan PEC. These chitosan-alginate PEC fibers are then freeze dried for further use [25]. Wet spun fibrous scaffolds also need to be optimized for their application in BTE, due to lower their degradation rate and increase mechanical strength.

### 2.6 HAp-derived inorganic coating

A natural polymer-based, highly porous scaffold with better mechanical strength and biological property is yet to be developed. In order to achieve this various surface techniques have been employed to produce different types of coatings on the surface of scaffolds[16]. Hydroxyapatite (HAp) being chemically similar to the apatite of human bone, HAp coating can be used to improve biologic function of the scaffold. Also, HAp is capable of forming tight bonds with the

host bone, it also facilitates cell adhesion, proliferation and differentiation. Conventionally, HAp is thought to be osteoconductive but reports have shown that with certain 3 dimensional geometries it is able to bind with endogenous bone morphogenic proteins and designated to be having osteoinductive properties[15]. It also improves the mechanical strength of the scaffold as a result of its crystal structure [27].

HA coatings can be produced by dip coating, spin coating or alternate soaking method, etc. Dip coating method involves immersion of scaffold in the HAp dispersion at a specific rate and withdrawal of the scaffold followed by evaporation of the solvent. Optimum thickness HAp coating can be achieved by repetitive cycles of dip coating [28].

Alternate soaking of scaffold in calcium chloride solution and disodium hydrogen phosphate solution also produce HAp coating. But size of HAp produced through this method ranges in micrometers. This results in significant decrease of the porosity [29]. Also, alternate soaking method is easy but time consuming method, whereas dip coating seems to be simple and quick method.

**2.7 Synthesis of nano-sized HAp**

There are several methods of preparing nano HAp reported in the literature, including wet chemical precipitation, biomimetic deposition, sol-gel and electrodeposition [30]. Wet chemical precipitation generally involves use of water as a solvent to mix calcium hydroxide and phosphoric acid to form HAp. Diammonium hydrogen phosphate and calcium nitrate have also been used to produce HAp through wet chemical precipitation. It has b7een reported that The rod-like shape HAP crystals with a diameter of ~ 6nm and length of 75 nm formed at room temperature in ethanol solvent[31].

# 3. AIMS & OBJECTIVES

The aim of this study is to develop a 3D porous hybrid scaffold using chitosan-alginate polyelectrolyte complex for bone tissue engineering applications & to modify the developed scaffold in order to achieve desired mechanical strength and bioactivity.

The specific objectives of this study are:

1. To develop a porous scaffold from chitosan-alginate polyelectrolyte complex (CAPEC),
2. To synthesize hydroxyapatite (HAp),
3. To coat the developed CAPEC scaffold with HAp in order to improve its mechanical strength and bioactivity,
4. To characterize the HAp coated CAPEC scaffold (CAPEC/HAp) for its pore size, porosity, mechanical strength, swelling behavior, in-vitro biodegradation,

# 4. MATERIALS & METHODS

## 4.1 Materials

### 4.1.1 Preparation of CAPEC scaffold

Chitosan (From Shrimp Shells, Degree of deacetylation >= 75%), Sodium alginate, Sodium hydroxide pellets, Acetic acid, Calcium chloride were purchased from Himedia, India. Sodium tripolyphospate was procured from Sigma Aldrich (USA).

### 4.1.2 Synthesis of Hydroxyapatite (HAp)

Commercially available Diammonium hydrogen phosphate ($(NH_4)_2HPO_4$) (Rankem), calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$) (Loba Chemie pvt. Ltd.), ammonia solution ($NH_4OH$) (Rankem), and absolute (anhydrous) ethanol (Sigma-Aldrich) were purchased.

## 4.2 Methods

### 4.2.1 Preparation of CAPEC scaffold

CAPEC scaffolds were prepared by the method reported elsewhere [8] with some minor changes. **Figure 4.2.1a** shows the flow chart for preparation of CAPEC scaffold. Briefly, Chitosan solution was prepared by dissolving chitosan powder (4.8 g) in 80 ml 1N acetic acid. 4.8 g of sodium alginate was dissolved in 120 ml 1N NaOH. The two solutions were mixed together using an overhead stirrer at 4000 rpm, under constant stirring. The homogenous chitosan-alginate (4.8% w/v) solution obtained contains 2.4% each chitosan and alginate. The CS-AG solution was

further homogenized at 4000 rpm for 15 minutes [21]. The diameter of dispersing element used was 25 mm. 2N acetic acid was used to adjust the pH of CS-AG solution to physiological pH (pH 7.4). The CS-AG solution was introduced into 6-well culture plates and maintained at -20$^0$C for 24 h. The samples were then freeze dried at -55$^0$C for 48 h. 1% $CaCl_2$ was used to crosslink the freeze dried scaffolds for 10-15 minutes. The scaffolds were washed thoroughly by distilled water to remove traces of unbound cross linker. The samples were then freeze dried.

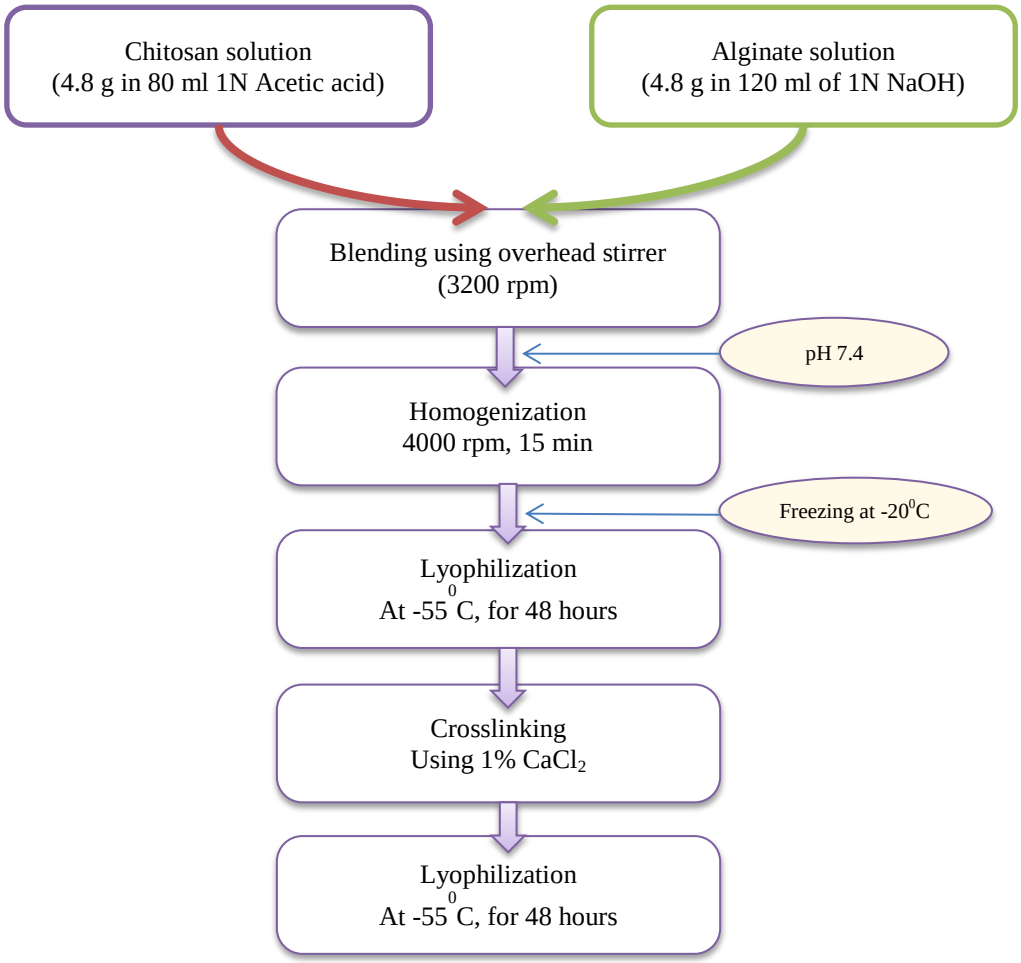


**Figure 4.2.1a** ***Flow chart of preparation of CAPEC scaffold***

### 4.2.2 Synthesis of hydroxyapatite (HAp)

Hydroxyapatite was synthesized using wet chemical precipitation method as follows [31]. First, 100 ml of 0.5 M $(NH_4)_2HPO_4$ & 0.3 M $Ca(NO_3)_2.4H_2O$ were prepared in ethanol. Before preparing the solution, each reactant was first dissolved in 5 ml of water followed by adjusting the pH between 10-12 using ammonia solution. Then the solutions were diluted with ethanol to make up the volume. 0.5 M $(NH_4)_2HPO_4$ solution was added to 0.3 M $Ca(NO_3)_2.4H_2O$ solution under constant stirring. The reaction was carried out at $40^0$C for 24 h. The solution was then filtered and washed, and then precipitate was collected and kept overnight in hot air oven at $40^0$C for drying.

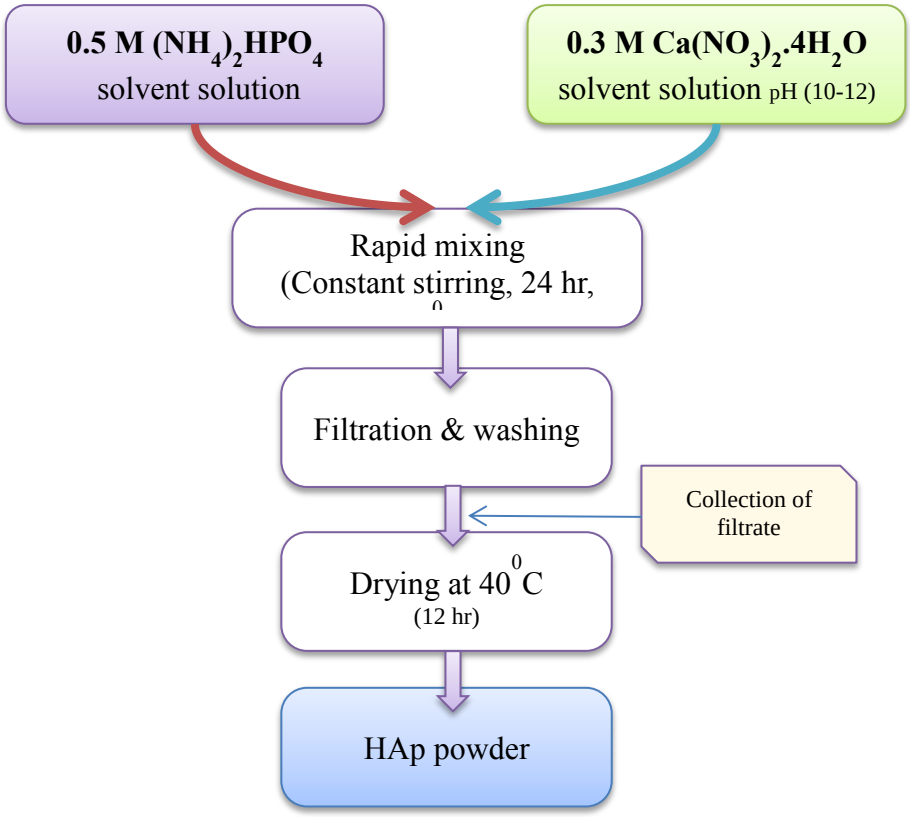


**Figure 4.2.2a** ***Schematic representation of methodology for synthesis of HAp***

### 4.2.3 Preparation of HAp coated-CAPEC scaffold (CAPEC/HAp)

HAp was deposited on CAPEC scaffold by dip coating method as described in literature [15]. The HAp suspension was prepared by dissolving 0.1 g HAp powder in 20 ml ethanol at room temperature. The suspension was maintained using ultrasonication bath for 1 hr. Then CAPEC scaffold was dipped in the dispersion slowly at the speed of 100mm/ minute for about 3 minutes. Then the scaffold was withdrawn from the HAp suspension and allowed to dry. For one batch of scaffolds, one more cycle of dip coating was performed. Scaffolds undergone only one cycle of dip coating were named as CAPEC/HAp1, whereas the scaffolds with two cycles of dip coating were designated as CAPEC/HAp2. The obtained CAPEC/HAp scaffolds were made ready for different characterization techniques.

## 4.3 Characterization

### 4.3.1 Average particle size

HAp sample was subjected to Dynamic Light Scattering (DLS) in order to find out average particle size. HAp powder was suspended in ethanol solution. The suspension was ultrasonicated for 30 minutes in an ultrasonication bath, before analysis.

### 4.3.2 Morphological characterization

The morphology of developed scaffolds was observed by SEM (Scanning Electron Microscopy). CAPEC scaffold and CAPEC/HAp scaffold samples were sputter-coated with Au/Pd and imaged by SEM (SEM JEOL-JSM 6480 LV). Along with Scaffold samples, the morphology of synthesized HAp samples was studied using SEM.

### 4.3.3 Porosity measurement

The overall porosity of the scaffold was measured using the fluid saturation method. A dry sample of known weight (Wd) was taken. Its bulk volume (Vb) was calculated. The sample was then immersed in water until it reaches saturation. Wet weight of the saturated scaffold was calculated as Ww. Volume of water (Vc) in the pores was measured by dividing the difference between wet weight and dry weight by density of water. The following equation was used to measure the scaffold porosity. Samples were tested in duplicates and the average was taken and calculated.

$$Porosity\ \% = \frac{Vc}{Vb} * 100$$

### 4.3.4 Phase analysis

Phase analysis of developed CAPEC scaffold , HAp powder and CAPEC/HAp composite scaffolds was performed by X-ray Diffractometer [PANalytical, X'pertPhilips,USA] using Cu-Kα radiation (λ= 0.1542 A°) at scanning rate of $10^0$/ minute with step size of $0.05^0$ within a scanning region of $2\theta = 10\text{-}50^0$. The operating conditions were 30 kV and 30 mA.

### 4.3.5 Functional analysis

Fourier transform infrared spectroscopy (FTIR) was performed to identify organic and inorganic compounds by determining the molecular composition and functional groups of the developed CAPEC scaffold, HAp powder and CAPEC/HAp composite scaffold. An Infra red Microscope [Shimadzu AIM-8800, Japan] was used for FTIR. The scaffold samples were pelletized using

hydraulic press by mixing them with dry KBr powder. The mixture was pressed into transparent disks and used for IR analysis. The machine was operated in transmittance mode by using the range 500 to 4000$cm^{-1}$ with a resolution of 8$cm^{-1}$..

### 4.3.6 Mechanical strength

Prepared CAPEC & CAPEC/HAp scaffolds were tested for their mechanical strength (compressive strength) using by Universal testing machine (H10 KS Tinius Olsen USA). Scaffolds were cut in square shape with a length of 7 mm and thickness of 8 mm for analysis. Compression test was performed with a crosshead speed of 1 mm/min with a load cell of 1kN until 60% compression was achieved. Compressive strength was calculated by using following formula;

$$s = (\frac{Fmax}{A})$$

Where, $F_{max}$ represents the force applied and A is the cross sectional area of the sample.

### 4.3.7 Swelling behavior

Swelling behavior of the developed CAPEC and CAPEC/Hap composite scaffolds was evaluated using deionised water at room temperature until they reach equilibrium. The dry weight of the scaffold was noted ($W_D$). Scaffolds were then placed in deionized water for different time intervals (1 h, 3 hrs, 5 hrs, 7 hrs, 24 hrs and 48 hrs). After each time interval, the scaffolds were withdrawn and surface adsorbed water was removed using filter paper and the wet weight was recorded ($W_T$). The swelling ratio and water uptake % was calculated using following equations,

$$Swelling\ ratio = \frac{WT - WD}{WD}$$

$$Water\ uptake\ \% = \frac{WT - WD}{WD} * 100$$

### 4.3.8 In-vitro biodegradation study

The scaffolds of known dry weights (Wi) were sterilized by immersing in 70% ethanol before starting the biodegradation study. Sterilized scaffold samples were then immersed in PBS (pH 7.4) at 37°C. The PBS solution was refreshed daily to ensure continuous degradation. The soaking time of the samples was 1, 3, 5, 7, 14, 21 and 28 days. The samples were removed at regular time intervals and vacuum dried before calculating the final weight, *Wf*. The extent of degradation was expressed as a percentage of weight remained of the dried sample after degradation. The percentage of weight remained was calculated using the following equation:

$$\%\ weight\ remaining\ =\ 100 - [\frac{Wi - Wf}{Wi} *\ 100]$$

# 5. RESULTS & DISCUSSION

This study focuses on the development of CAPEC scaffold for the bone tissue engineering application. The intention was to achieve desired pore size, porosity, mechanical strength, swelling behavior, biodegradation rate. The prepared scaffolds were characterized for all these required parameters. In order to improve the mechanical strength and bioactivity of the developed CAPEC scaffold, they were coated with HAp. The HAp was synthesized by wet chemical precipitation method. To confirm the synthesis and crystallinity of HAp, characterization of prepared sample was done. Also, particle size of the synthesized HAp was determined in order to confirm the nano-sized HAp formation. This chapter describes results and discussion on this study.

### 5.1 Average particle size

Average particle size of synthesized HAp was studied using DLS. The average particle size of HAp was found to be 159.2 nm. **Figure 5.1.1** shows the size distribution of HAp by intensity.

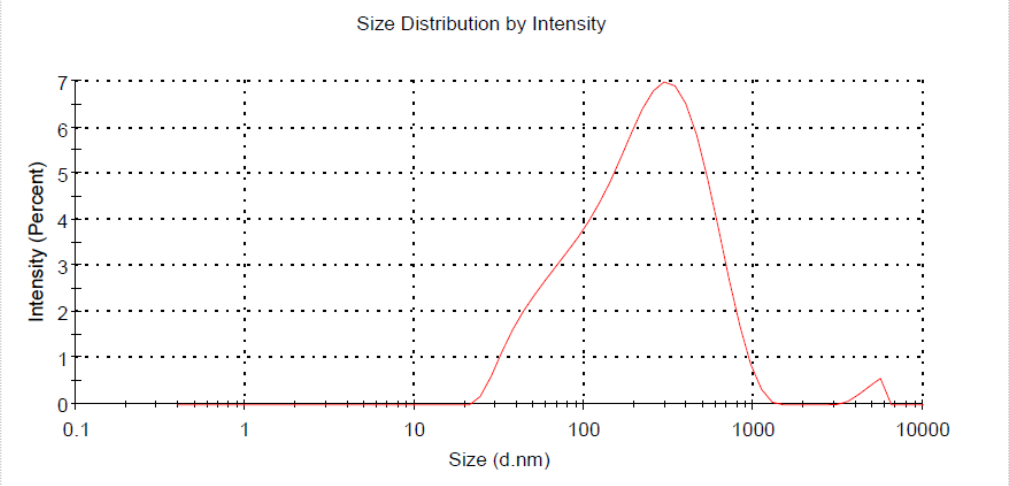


**Figure 5.1.1** ***Size distribution profile of HAp sample***

## 5.2 Preparation of scaffolds

3D porous scaffolds from chitosan-alginate polyelectrolyte complex were prepared by freeze drying technique. Using dip coating method, prepared scaffolds were coated with nano-HAp. Scaffolds were designated as CAPEC (CAPEC cross-linked with $CaCl_2$), CAPEC/HAp1 (one cycle of coating) and CAPEC/HAp2 (two cycles of coating). The scaffolds developed are shown in the following **figure 5.2.1**.

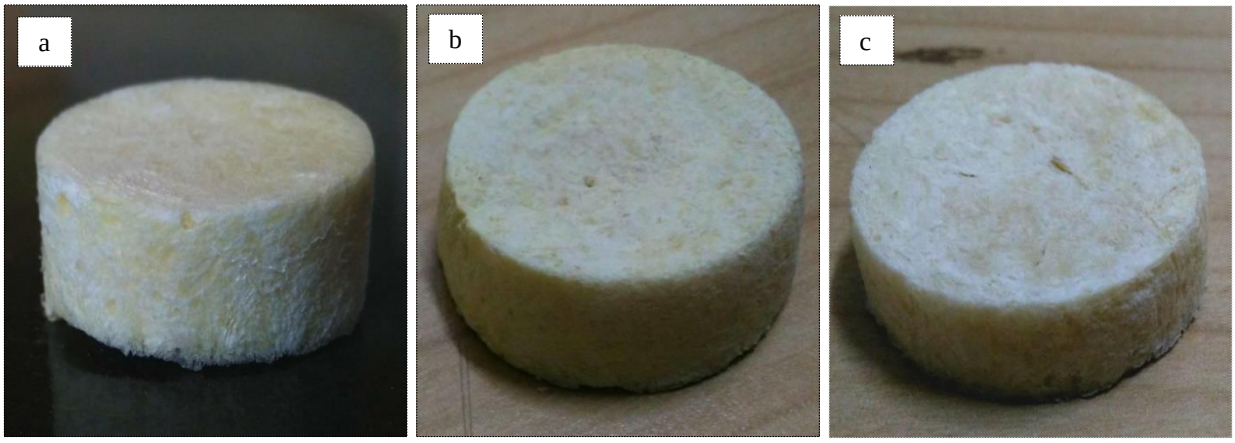


**Figure 5.2.1** ***a) Developed CAPEC scaffold, b) CAPEC/HAp1 scaffold, c) CAPEC/HAp2 scaffold***

## 5.3 Morphology & pore size

SEM analysis was performed on developed scaffolds. SEM images of developed scaffolds are shown in the **figure 5.3.1**. CAPEC scaffold showed interconnected open pore structure. The porous network is composed of both micro and micro pores, which is found to be optimum for bone tissue engineering. The pore size ranged from ~30 to 280µm. SEM images of CAPEC/HAp1 and CAPEC/HAp2 scaffold shows the presence of HAp on the walls of scaffold.

But the distribution of HAp was not uniform due to the presence of agglomerates of HAp. There was no apparent change in the pore size of the scaffold.

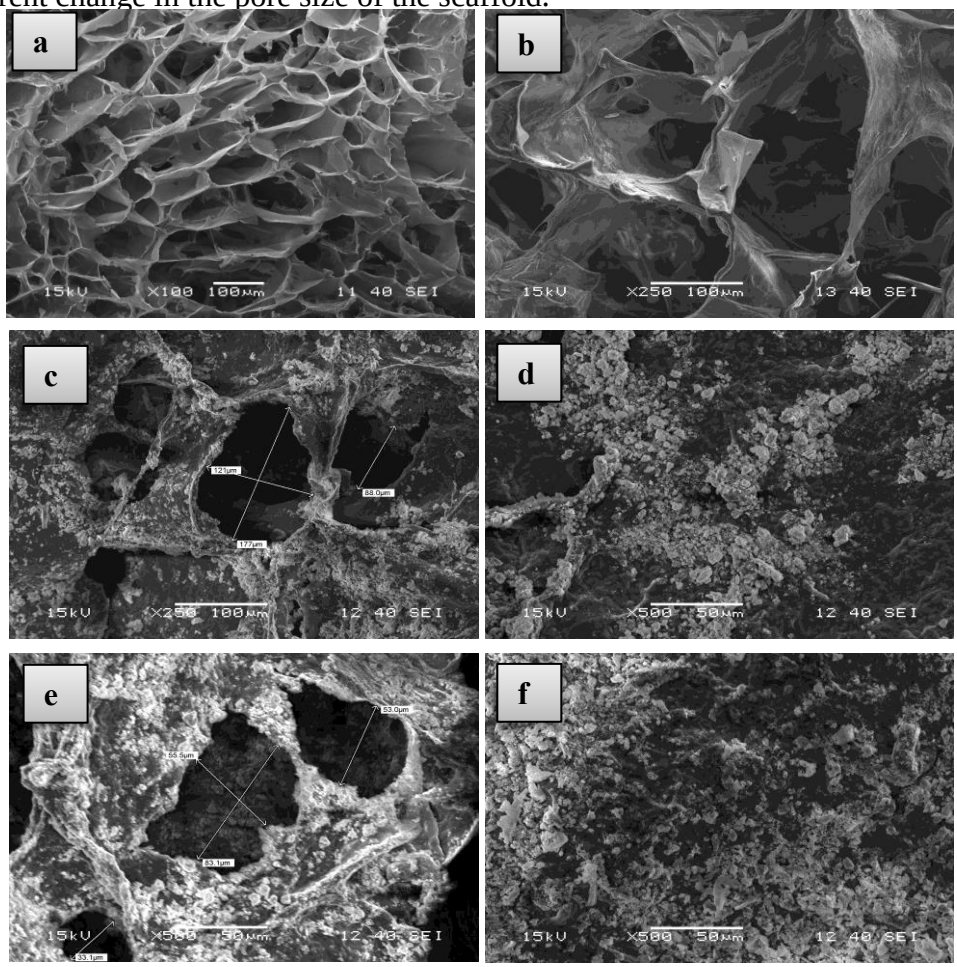


**Figure 5.3.1** ***SEM images of CAPEC scaffold (a & b), CAPEC/HAp1 scaffold (c & d) and CAPEC/HAp2 scaffold (e & f)***

### 5.4 Porosity

An ideal scaffold must have interconnected pores which facilitate the transport of cells, nutrients, metabolites. % porosity of the developed CAPEC scaffold was found to be ~82% on an average. Decrease in porosity was found in case of HAp coated CAPEC scaffolds. CAPEC/HAp1 and CAPEC/HAp2 scaffold showed average porosity of ~76% and ~69%, respectively. This descrease in porosity may be attributed to the deposition of HAp on the walls of scaffold.

However, the porosity of the CAPEC/HAP1 scaffold is still high enough for the bone tissue engineering application.

### 5.5 Phase analysis

Phase changes, if any, in CAPEC scaffold due to crosslinking and HAp coating were analyzed by XRD studies. **Figure 5.5.1** shows X-ray diffraction patterns for CAPEC scaffold (before crosslinking, CS-AG), CAPEC scaffold (after crosslinking), HAp, CAPEC/HAp1 scaffold. XRD pattern of CS-AG shows sharp peaks corresponding to chitosan-alginate interaction. Whereas, for crosslinked CAPEC scaffold, peaks are broader and the corresponding peak intensity is decreased. For HAp powder, presence of sharp peaks at $2\theta = 25.8^0$, $31.8^0$, $32.1^0$, $32.9^0$, $34.0^0$ corresponds to its crystalline nature. For CAPEC/HAp scaffold, the peaks of chitosan-alginate and HAp are present and show crystalline nature but the peaks are weaker and broader.

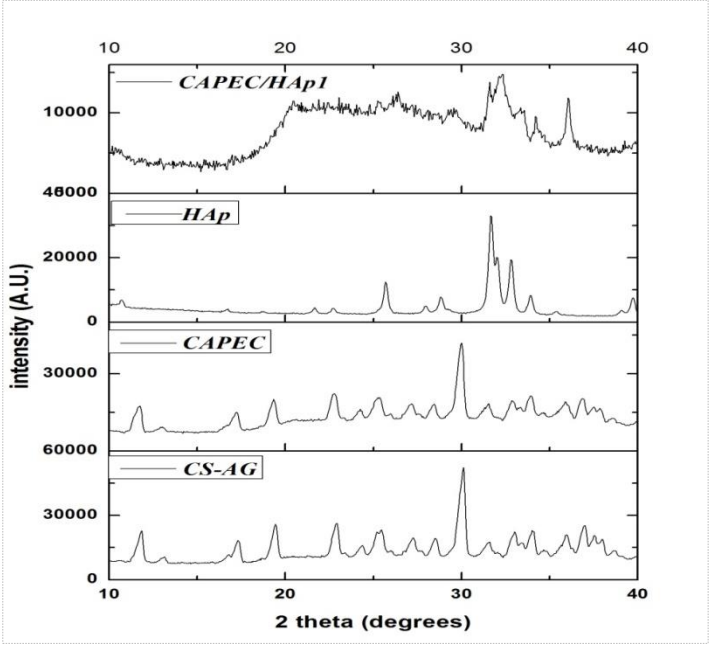


**Figure 5.5.1** ***XRD spectra of CS-AG, CAPEC scaffold, HAp powder and CAPEC/HAp1 Scaffold***

## 5.6 Functional analysis

**Figure 5.6.1** shows the IR spectra of pure chitosan, pure alginate, HAp and CAPEC/HAp Scaffold. IR spectra of pure chitosan shows the characteristic bands of amino group (1173cm$^{-1}$ ) and amide group (1643cm-1). Alginate spectrum shows a peak at 1607 cm$^{-1}$ specific for carbonyl bond. In the IR spectra of chitosan and alginate, peaks at 1075 cm$^{-1}$ and 1424 cm$^{-1}$ corresponding to carboxyl –COOH and C-O stretching can be seen. HAp spectrum shows band at 603 cm$^{-1}$ which signifies the vibration of hydroxyl ions, whereas bands at 1034 and 565 cm$^{-1}$ corresponds to phosphate bending in HAp. In the spectra of CAPEC/HAp, peak shift of Amide group from 1643 to 1607 cm$^{-1}$ is seen, while the amine group peak is absent. The shift in the amide group peak and absence of amino group peak is may be due to the chitosan-alginate polyelectrolyte interaction between the carboxyl and amine group of chitosan and alginate.

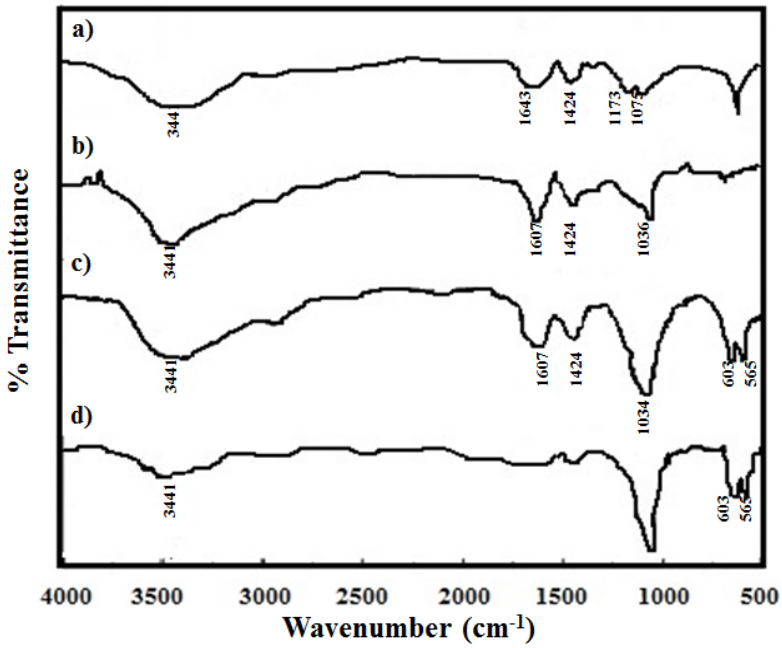


**Figure 5.6.1** ***FTIR spectra of a) pure chitosan, b) pure alginate, c) CAPEC/HAp1 scaffold and d) HAp powder.***

## 5.7 Mechanical strength

Until the tissue is regenerated, a scaffold must provide mechanical integrity, it should act as a support against the stress generated by new tissue formation. The developed scaffolds were tested for their compressive strength. **Figure 5.7.1** depicts the compressive strength of CAPEC scaffold (Un-crosslinked, CS-AG), CAPEC scaffold (crosslinked), CAPEC/HAp1 scaffold and CAPEC/HAp2 scaffold. After crosslinking with CaCl2, compressive strength of CAPEC scaffold is increased from 0.23 MPa to 0.469 MPa. This increase in mechanical strength can be attributed to the strong ionic interaction of $Ca^{+2}$ with $COO^{-}$ of alginate chain[8]. Similarly, it is seen that HAp coating also resulted in increase in compressive strength of the scaffold [27]. Compressive strength for CAPEC/HAp1 and CAPEC/HAp2 scaffolds was found to be 0.61 MPa and 0.67 MPa, respectively. Increase in compressive strength can also be related to decrease in porosity due to distribution of HAp along the walls of CAPEC scaffold.

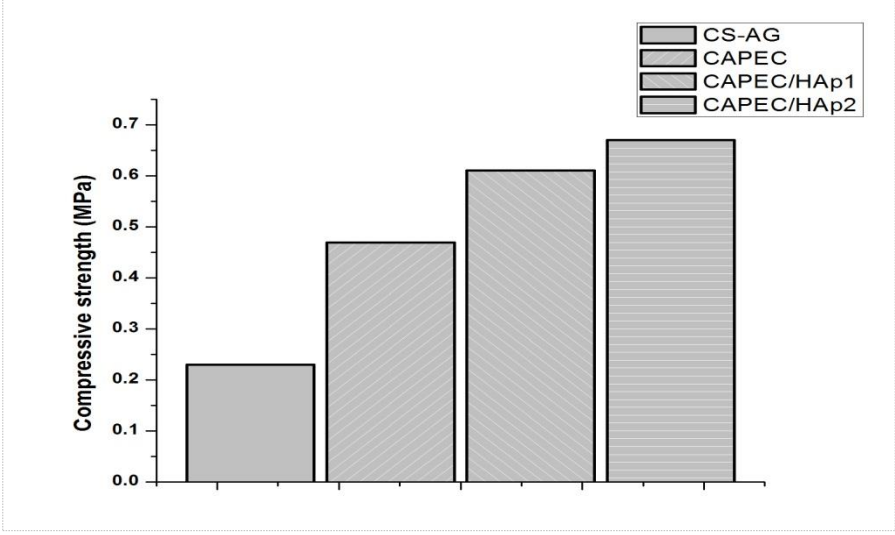


**Figure 5.7.1** ***Compressive strength (MPa) of CS-AG, CAPEC, CAPEC/HAp1 and CAPEC/HAp2 Scaffolds***

### 5.8 Swelling behavior

**Figure 5.8.1** shows the shape retention of each scaffold sample after 2 days of incubation. Swelling behavior of developed scaffolds; CAPEC, CAPEC/HAp1 and CAPEC/HAp2 scaffold is shown in the following **figure 5.8.2**. It can be seen that the CAPEC scaffolds shows higher swelling percentage (~450%) than the HAp coated CAPEC scaffolds (~330% and ~250%). It is observed that the swelling % is decreased with the increase in HAp coating. The swelling percentage for CAPEC/HAp1 scaffolds was found to be ~330%, whereas for CAPEC/HAp2 scaffolds it was found to be ~250%. This may be attributed to decrease in porosity as well as decrease in the diffusion of water due to HAp depositions on the wall of scaffolds.

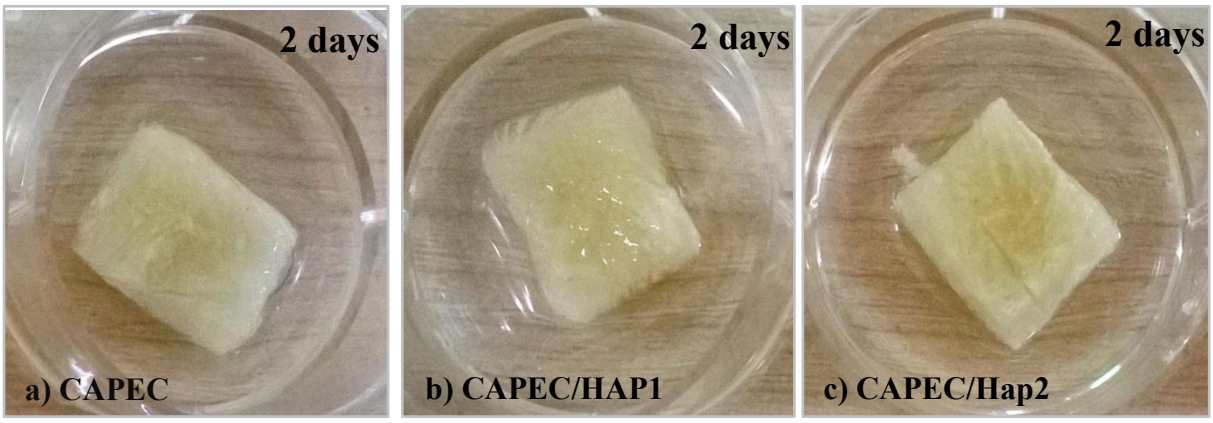


**Figure** ***5.8.1 Images of CAPEC (a), CAPEC/HAp1 (b) and CAPEC/HAp2 (C) scaffolds after two days of immersion in PBS.***

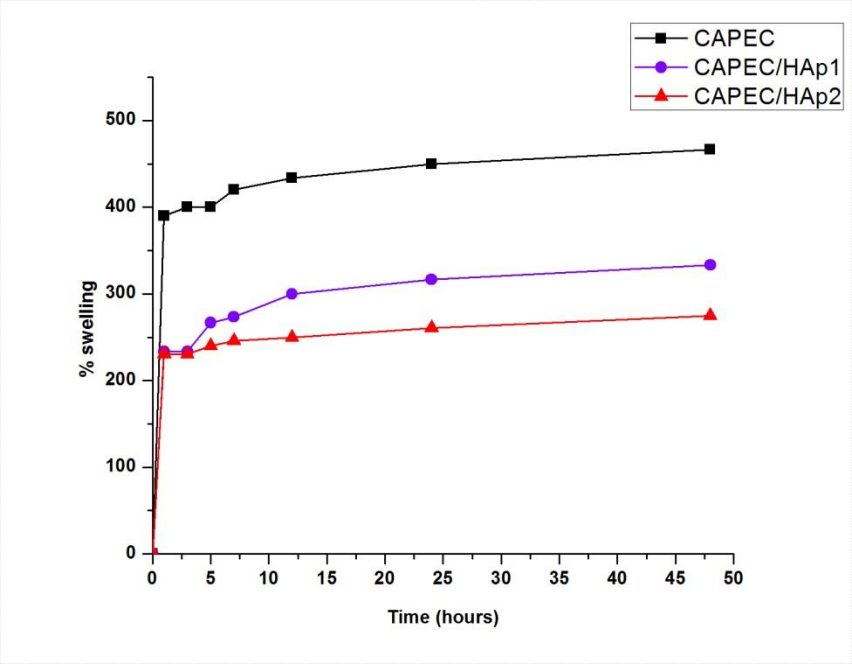


**Figure 5.8.2** ***Percentage swelling of scaffolds as a function of sample immersion time in PBS: CAPEC, CAPEC/HAp, CAPEC/HAp2 Scaffolds.***

### 5.9 In-vitro biodegradation

For an ideal scaffold, rate of biodegradation must be equal to the rate of new tissue formation. In order to achieve this, control of biodegradation rate of a scaffold is necessary. Developed scaffolds were subjected to in-vitro biodegradation for a period of 28 days. **Figure 5.9.1** show the % loss in weight of the scaffold samples as a function of immersion time in PBS. It was seen that the rate of degradation in case of HAp coated CAPEC scaffold was lower than that for the CAPEC scaffold. Following figure shows the % weight remaining of the scaffold as a function of time. CAPEC scaffold lost almost 32% of the weight during the period of study, whereas for CAPEC/HAp1 and CAPEC/HAp2 scaffolds the % weight loss was ~30% and ~ 22%. Difference in rate of biodegradation may correspond to increased mechanical stability due to HAp coating.

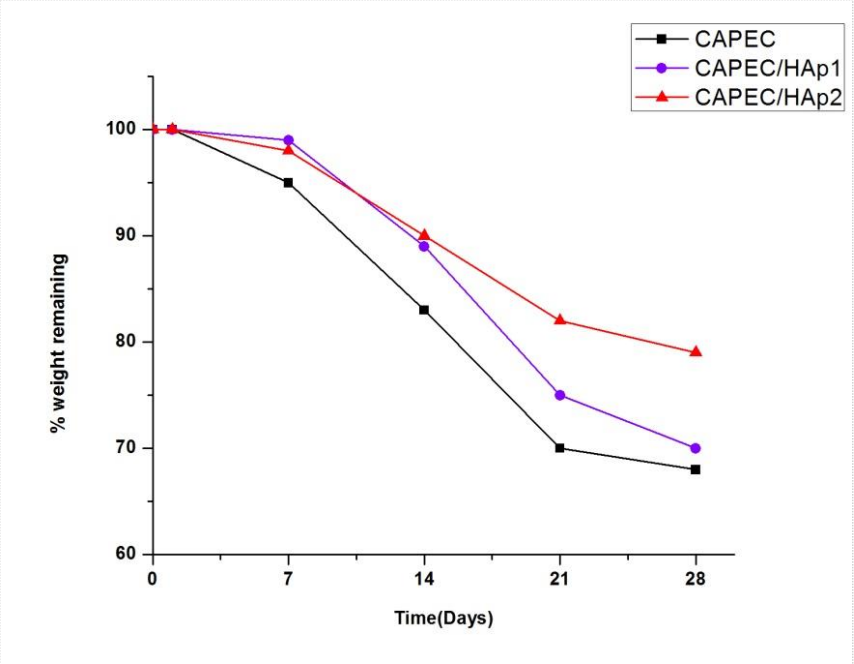


**Figure 5.9.1** ***Percentage weight remaining of scaffolds as a function of sample immersion time in PBS: CAPEC, CAPEC/HAp, CAPEC/HAp2 Scaffolds.***

# 6. SUMMARY & CONCLUSION

In the present study, 3D porous scaffolds using chitosan-alginate polyelectrolyte complex were developed by freeze drying method. The developed scaffolds were further modified by employing dip coating with the HAp. The HAp was synthesized by wet chemical precipitation method. Instead of using water as a solvent, ethanol was used as a solvent for dissolution of the reactants. Average particle analysis of synthesized HAp shows the average particle size of HAp as ~159.2 nm. XRD analysis of HAp also confirms the crystalline nature of synthesized HAp. CAPEC scaffolds coated once with HAp were found to be optimum for non-load bearing bone tissue engineering applications. SEM analysis revealed the similar morphology and pore size of CAPEC and CAPEC/HAp scaffolds with interconnected pore network made up of both micro and macropores (~44 to ~180µm). But the porosity was decreased with the increase in HAp concentration. There was a decrease in porosity from ~82% to ~69% in case of CAPEC/HAp scaffolds. But CAPEC/HAp1 scaffolds displayed ~76% porosity, which is high enough for tissue engineering applications. Increase in the mechanical strength was observed in CAPEC scaffolds after the coating with HAp. Compressive strength of CAPEC scaffolds was found to be 0.469 MPa, whereas in case of CAPEC/HAp1 and CAPEC/HAp2 scaffolds it was found to be increased to 0.61 and 0.67MPa. The swelling behavior and in-vitro biodegradation studies also suggest that the developed scaffolds are favorable. In conclusion, the developed CAPEC/HAp can be used as a bone scaffold in case of soft bone. In future, the developed hybrid composite scaffold might provide a promising alternative to bone grafts in bone tissue engineering.

# REFERENCES


1. Amini, A.R., C.T. Laurencin, and S.P. Nukavarapu, *Bone tissue engineering: recent advances and challenges.* Critical Reviews™ in Biomedical Engineering, 2012. **40**(5).
2. Salgado, A.J., O.P. Coutinho, and R.L. Reis, *Bone tissue engineering: state of the art and future trends.* Macromolecular bioscience, 2004. **4**(8): p. 743-765.
3. Costa-Pinto, A.R., R.L. Reis, and N.M. Neves, *Scaffolds based bone tissue engineering: the role of chitosan.* Tissue Engineering Part B: Reviews, 2011. **17**(5): p. 331-347.
4. Nguyen, D.T. and K.J. Burg, *Bone tissue engineering and regenerative medicine: Targeting pathological fractures.* Journal of Biomedical Materials Research Part A, 2015. **103**(1): p. 420-429.
5. Sabir, M.I., X. Xu, and L. Li, *A review on biodegradable polymeric materials for bone tissue engineering applications.* Journal of Materials Science, 2009. **44**(21): p. 5713-5724.
6. Stroncek, J.D. and W.M. Reichert, *Overview of wound healing in different tissue types.* Indwelling neural implants: strategies for contending with the in vivo environment, 2008.
7. Mravic, M., B. Péault, and A.W. James, *Current trends in bone tissue engineering.* BioMed research international, 2014. **2014**.
8. Li, Z., et al., *Chitosan–alginate hybrid scaffolds for bone tissue engineering.* Biomaterials, 2005. **26**(18): p. 3919-3928.
9. Han, J., et al., *Alginate–chitosan/hydroxyapatite polyelectrolyte complex porous scaffolds: Preparation and characterization.* International journal of biological macromolecules, 2010. **46**(2): p. 199-205.
10. Cheema, U., *Regenerative medicine and tissue engineering-Cells and biomaterials.* 2011.
11. Venkatesan, J. and S.-K. Kim, *Chitosan composites for bone tissue engineering—An overview.* Marine drugs, 2010. **8**(8): p. 2252-2266.
12. Bose, S., M. Roy, and A. Bandyopadhyay, *Recent advances in bone tissue engineering scaffolds.* Trends in biotechnology, 2012. **30**(10): p. 546-554.
13. Venkatesan, J., et al., *Alginate composites for bone tissue engineering: A review.* International journal of biological macromolecules, 2015. **72**: p. 269-281.
14. Hamman, J.H., *Chitosan based polyelectrolyte complexes as potential carrier materials in drug delivery systems.* Marine Drugs, 2010. **8**(4): p. 1305-1322.
15. Goodman, S.B., et al., *The future of biologic coatings for orthopaedic implants.* Biomaterials, 2013. **34**(13): p. 3174-3183.
16. Wu, S., et al., *Biomimetic porous scaffolds for bone tissue engineering.* Materials Science and Engineering: R: Reports, 2014. **80**: p. 1-36.
17. Shrivats, A.R., M.C. McDermott, and J.O. Hollinger, *Bone tissue engineering: state of the union.* Drug discovery today, 2014. **19**(6): p. 781-786.
18. Archana, D., et al., *Chitosan–pectin–alginate as a novel scaffold for tissue engineering applications.* Indian J Biotechnol, 2013. **12**: p. 475-482.
19. Di Martino, A., M. Sittinger, and M.V. Risbud, *Chitosan: a versatile biopolymer for orthopaedic tissue-engineering.* Biomaterials, 2005. **26**(30): p. 5983-5990.
20. Lee, K.Y. and D.J. Mooney, *Alginate: properties and biomedical applications.* Progress in polymer science, 2012. **37**(1): p. 106-126.
21. He, B., M. Leung, and M. Zhang, *Optimizing creation and degradation of chitosan-alginate scaffolds for in vitro cell culture.*

22. Lee, S.H., et al., *Osteogenic activity of chitosan-based hybrid scaffold prepared by polyelectrolyte complex formation with alginate.* Tissue Engineering and Regenerative Medicine, 2014. **11**(2): p. 106-112.
23. Florczyk, S.J., et al., *3D porous chitosan–alginate scaffolds: a new matrix for studying prostate cancer cell–lymphocyte interactions in vitro.* Advanced healthcare materials, 2012. **1**(5): p. 590-599.
24. Jin, H.-H., et al., *In-situ formation of the hydroxyapatite/chitosan-alginate composite scaffolds.* Materials Letters, 2008. **62**(10): p. 1630-1633.
25. Shao, X. and C.J. Hunter, *Developing an alginate/chitosan hybrid fiber scaffold for annulus fibrosus cells.* Journal of Biomedical Materials Research Part A, 2007. **82**(3): p. 701-710.
26. Wang, J.-Z., et al., *Spray-spinning: a novel method for making alginate/chitosan fibrous scaffold.* Journal of Materials Science: Materials in Medicine, 2010. **21**(2): p. 497-506.
27. Kim, H.-L., et al., *Preparation and characterization of nano-sized hydroxyapatite/alginate/chitosan composite scaffolds for bone tissue engineering.* Materials Science and Engineering: C, 2015.
28. Furuzono, T., et al., *Preparation and characterization of apatite deposited on silk fabric using an alternate soaking process.* Journal of biomedical materials research, 2000. **50**(3): p. 344-352.
29. Li, H., et al., *Effect of Thickness of HA-Coating on Microporous Silk Scaffolds Using Alternate Soaking Technology.* BioMed research international, 2014. **2014**.
30. Ferraz, M., F. Monteiro, and C. Manuel, *Hydroxyapatite nanoparticles: a review of preparation methodologies.* Journal of Applied Biomaterials and Biomechanics, 2004. **2**(2): p. 74-80.
31. Alobeedallah, H., et al., *Preparation of Nanostructured Hydroxyapatite in Organic Solvents for Clinical Applications.* Trends in Biomaterials and Artificial Organs, 2011. **25**(1): p. 12-19.